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NOTES

Newly Isolated Strains of *Rickettsia tsutsugamushi* in Japan Identified by Using Monoclonal Antibodies to Karp, Gilliam, and Kato Strains

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Four isolated strains of *Rickettsia tsutsugamushi* from patients in a new endemic area of Japan were tested for antigenicities by using 12 monoclonal antibodies to Karp, Gilliam, and Kato strains. It was suggested that one isolate was Karp related and that the others were two independent strains.

Since 1976, the incidence of tsutsugamushi disease has increased in Japan (9). It had never been reported in Gifu Prefecture, located near the center of Japan's main island of Honshu, until 1981. After 1982, the recorded incidence of the disease in this prefecture was 5th to 7th highest (26 to 52 patients per year) in Japan. We isolated four strains of *Rickettsia tsutsugamushi* from patients in this area to investigate the nature of these strains, not only to detect the prevalent strains in this area but also to speculate on the real cause of the sudden emergence of the disease in this prefecture. Recent studies suggested the usefulness of murine monoclonal antibodies (MAbs) to standard strains (Karp, Gilliam, and Kato) of *R. tsutsugamushi* for classifying the isolates (1, 2, 7). In this study, the antigenicities of the new isolates were analyzed by using either strain-specific or cross-reactive MAbs to standard strains.

The Karp, Gilliam, and Kato strains of *R. tsutsugamushi* were kindly supplied from the Toyama Prefectural Institute of Public Health, Toyama, Japan. Four newly isolated strains from tsutsugamushi disease patients in Gifu Prefecture (Japan) (3) were propagated in BS-C-1 cells. Maintenance of these strains was performed as described by Tamura et al. (10).

Five- to six-week-old male BALB/c mice were infected either subcutaneously (for Karp and Kato strains) or intraperitoneally (for Gilliam strain) with 100 times the mouse 50% lethal dose of each strain. After 5 to 6 weeks, the mice were inoculated intravenously with 1,000 times the mouse 50% lethal dose of the same rickettsial strain. Lymphocyte hybridomas were prepared 7 days later by fusing the spleen and NS-1 myeloma cells by using polyethylene glycol (Koch-Light Laboratories Ltd., Colnbrook, England), as described by others (6, 8). Hybridomas secreting rickettsial antibodies were identified by using the indirect fluorescent-antibody test (IFA) in which culture fluids were reacted with spotted infected cells and fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G (heavy and light chain) goat serum (Cooper Biomedical, Inc., West Chester, Pa.). These hybridomas were cloned two to three times by limiting dilutions. Rickettsial antibody-secreting hybridomas (10^7 cells) were injected into pristane (Aldrich Chemical Co., Inc., Madison, Wis.)-primed BALB/c nude mice. The immunoglobulin class of the MAb was determined by the Ouchterlony method using anti-μ, anti-γ1, anti-γ2a, anti-γ2b, and anti-γ3 serum (Cooper Biomedical). The immune ascites were titrated to standard and isolated strains of *R. tsutsugamushi* by IFA.

Twelve clones of hybridomas that secreted MAbs to standard strains of *R. tsutsugamushi* were established. The reactivity of each MAb is shown in Table 1. Strain-specific MAbs (Kp/D11, Gi/E4, Kt/1D2, and Kt/2D9) were identified by IFA titers against homologous antigens ranging from 1/320 to 1/20,480 and titers of less than 1/10 against heterologous antigens. Two MAbs (Kp/C6 and Kt/3B2) exhibited similar IFA titers with all three standard strains. Kp/1C10 exhibited IFA titers of 1/1,280 against a homologous strain and 1/80 against heterologous strains. Five MAbs reacted with a homologous strain plus one of the others, i.e., three MAbs (Kp/1F11, Kt/4D9, and Kt/3C2) reacted with both Karp and Kato strains. One MAb (Kp/2B7) reacted with both Karp and Gilliam strains, and another (Gi/E2) reacted with both Gilliam and Kato strains.

Four isolated strains (KN-1, KN-2, KN-3, and KN-4) were examined for reactivities to 12 MAbs by IFA to classify their antigenicities (Table 1). Strain KN-3 reacted with Karp-specific Kp/D11 and Karp-Kato-reactive Kp/1F11 and Kt/3C2. However, strain KN-3 reacted with neither Karp-Gilliam-reactive Kp/2B7 nor Karp-Kato-reactive Kt/4D9. KN-1 was the only strain which did not react with cross-reactive Kp/1C10. Furthermore, strain KN-1 reacted slightly with Karp-specific Kp/D11 and Gilliam-specific Gi/E4 but not with Kato-specific MAb. Strains KN-2 and KN-4 were suspected of being identical because they exhibited almost the same titers against all MAbs tested. These strains had reactivities to cross-reactive MAbs (Kp/1F11, Kp/1C10, Kp/C6, and Kt/3B2) with high titers and Karp-specific and Kato-specific MAbs (Kp/D11 and Kt/1D2) with low titers.

The usefulness of MAbs to *R. tsutsugamushi* strains for classifying their antigenicities was suggested by Eisemann and Osterman (1), Murata et al. (7), and Kanemitsu (2). In the present study, we established 12 strain-specific and

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TABLE 1. Reactivities of MAbs to standard and isolated *R. tsutsugamushi* strains

Clone code	Immunoglobulin	IFA titer[a]						
		Standard strains			Isolated strains			
		Karp	Gilliam	Kato	KN-1	KN-2	KN-4	KN-3
Kp/D11	M	640	<[b]	<	20	20	20	160
Kp/1F11	G3	2,560	<	80	2,560	2,560	2,560	320
Kp/2B7	G2a	80	80	<	<	<	<	<
Kp/1C10	M	1,280	80	80	<	640	1,280	1,280
Kp/C6	G2a	1,280	1,280	1,280	640	640	640	40
Gi/E4	G2a	<	10,240	<	10	<	<	<
Gi/E2	G2a	<	80	20	<	<	<	<
Kt/1D2	G2a	<	<	320	<	10	10	<
Kt/2D9	G2a	<	<	20,480	<	<	<	<
Kt/4D9	G1	20	<	640	<	<	<	<
Kt/3C2	G2b	2,560	<	2,560	<	<	<	2,560
Kt/3B2	G2a	2,560	2,560	2,560	640	2,560	2,560	640

[a] Reciprocal of the highest dilution of immune ascitic fluid causing rickettsial fluorescence.
[b] <, Less than 1:10.

cross-reactive MAbs to standard strains of *R. tsutsugamushi*. We were able to identify four newly isolated strains (KN-1, KN-2, KN-3, and KN-4). These isolates fell into three groups by using the IFA with MAb, and only one (KN-3) was related to the Karp strain. Strain KN-3 differed from the Karp strain, however, by having low reactivity (Kp/C6) and nonreactivity (Kp/2B7 and Kt/4D9) with MAbs to which the Karp strain reacted with high titers. These results suggested that KN-3 was an independent strain, although it is possible that the isolated strain or KN-3 differed from the laboratory-maintained strain only by epitope density. Strains KN-1 and KN-2 (=KN-4) were suggested to be independent strains because of low or no reactivities against strain-specific MAbs. Moreover, reactivity against cross-reactive Kp/1C10 was critical between the two strains.

In recent years, several investigators reported new isolates which were antigenically distinguished from standard strains (2, 5, 11). Kobayashi et al. (4) reported newly isolated strains which were identified as the Gilliam-Kato type by using the IFA technique with MAbs to Karp, Gilliam, and Kato strains. It was surprising to see that three independent strains were isolated from four patients in such a small geographical area and that all isolates revealed antigenic differences from standard strains.

Further investigation should focus on an analysis of the antigenicities of these isolated strains, along with their pathogenicities.

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